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Cardiac Hepcidin Expression Associates with Injury Independent of Iron

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Key Words
Cardiac hepcidin gene expression · Renocardiac failure · Iron

Abstract
Background: Hepcidin regulates systemic iron homeostasis by downregulating the iron exporter ferroportin. Circulating hepcidin is mainly derived from the liver but hepcidin is also produced in the heart. We studied the differential and local regulation of hepcidin gene expression in response to myocardial infarction (MI) and/or chronic kidney disease (CKD). We hypothesized that cardiac hepcidin gene expression is induced by and regulated to severity of cardiac injury, either through direct (MI) or remote (CKD) stimuli, as well as through increased local iron content. ***Methods:*** Nine weeks after subtotal nephrectomy (SNX) or sham surgery (CON), rats were subjected to coronary ligation (CL) or sham surgery to realize 4 groups: CON, SNX, CL and SNX + CL. In week 16, the gene expression of hepcidin, iron and damage markers in cardiac and liver tissues was assessed by quantitative polymerase chain reaction and ferritin protein expression was studied by immunohistochemistry. ***Results:*** Cardiac hepcidin messenger RNA (mRNA) expression was increased 2-fold in CL ($p = 0.03$) and 3-fold in SNX ($p = 0.01$). Cardiac ferritin staining was not different among groups. Cardiac hepcidin mRNA expression correlated with mRNA expression levels of brain natriuretic peptide ($\beta = 0.734$, $p < 0.001$) and connective tissue growth factor ($\beta = 0.431$, $p = 0.02$). In contrast, liver hepcidin expression was unaffected by SNX and CL alone, while it had decreased 50% in SNX + CL ($p < 0.05$). Hepatic ferritin immunostaining was not different among groups. ***Conclusions:*** Our data indicate differences in hepcidin regulation in liver and heart and suggest a role for injury rather than iron as the driving force for cardiac hepcidin expression in renocardiac failure.

Introduction

Iron-dependent modulation of energy metabolism is important in tissues with high metabolic demand, such as the myocardium [1]. The mechanisms involved in local

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iron metabolism are of critical importance since both iron overload and iron deficiency have poor outcomes in the heart [2–4]. Hepcidin is the main regulatory protein of systemic iron metabolism. It is mainly produced in the liver and binds to ferroportin-1 (Fpn-1), a cellular iron exporter, resulting in internalization and degradation of the complex [5]. Hepcidin thus inhibits cellular iron efflux from enterocytes, macrophages and hepatocytes [6]. Because hepcidin synthesis is primarily controlled at the transcriptional level [7], hepcidin antimicrobial peptide (Hamp) messenger RNA (mRNA) expression level is a good indication of the amount of hepcidin peptide produced.

Circulating hepcidin levels are mainly derived from the liver. Hepcidin is also expressed by other cells, such as the heart [8, 9], albeit at a much lower level by comparison [10]. Locally produced hepcidin affects iron homeostasis in an autocrine fashion, as shown by the downregulation of Fpn-1 expression by hepcidin produced by inflammatory monocytes [11]. In vitro, hepcidin reduces Fpn-1 content and iron release in cardiomyocytes [12], suggesting that hepcidin is involved in regulating cardiac iron turnover. Previous studies show that myocardial ischemia induces cardiac hepcidin expression and consequently enhances ferritin content in cardiomyocytes, this being related to the severity of ischemia [8, 9]. This suggests that, in addition to systemic regulation, hepcidin expression is also regulated at organ level and may influence cardiac iron content in response to myocardial injury. Indeed, cardiac hepcidin expression is regulated by a number of factors including hypoxia and inflammation [8].

Recently, we developed a rodent model of chronic renocardiac failure by subtotal nephrectomy (SNX) followed by coronary ligation (CL) [13]. CL superimposed on SNX leads to more severe heart failure. For the current study, we assessed cardiac and hepatic hepcidin mRNA expression and related regulatory genes as well as ferritin protein expression, an indicator of local iron stores in this model.

The present study investigated the assumption that hepcidin expression in liver and heart is differentially regulated. We hypothesized that cardiac hepcidin is upregulated in response to damage, both in models that induce damage to the myocardium directly (i.e., myocardial infarction (MI) induced by CL) as well as in models that damage the myocardium indirectly (i.e., chronic kidney disease (CKD) induced by renal ablation). Furthermore, we hypothesized that the induction of hepcidin gene expression is related to the severity of cardiac injury and increases local iron content.

Methods

Study Design

The study protocol was approved by the Ethics Committee on Animal Experiments of the University of Utrecht, Utrecht, The Netherlands. Male inbred Lewis rats (Lew/Crl; 180–200 g) were purchased from Charles River, Germany, and housed in a climate-controlled facility with a 12:12-hour light:dark cycle. From t = –1 to week 0, a 2-stage SNX by resection or sham operation was performed as described previously [13]. Briefly, first the right kidney was removed, and 1 week later, the poles of the left kidney were cut off. Adequacy of the SNX procedure was confirmed by an increase in plasma urea. From t = 1 week onward, rats received standard powdered chow supplemented with 6% NaCl until the end of the study. In Lewis rats, high salt intake is required to induce fluid overload and hypertension after SNX [13]. At t = 9 weeks, rats from both groups were either subjected to CL or sham operation. This resulted in 4 groups: CON (sham-SNX + sham-CL, n = 10), SNX (SNX + sham-CL, n = 12), CL (sham-SNX + CL, n = 9) and SNX + CL (n = 9). Rats were followed up to week 16. To assess end diastolic volume (EDV), transthoracic echocardiography was performed with a digital ultrasound machine (model Sonos 5500, Philips Research, Eindhoven, The Netherlands) and a 15-MHz linear array transducer (Hewlett Packard, Palo Alto, Calif., USA). CL and SNX + CL rats without visible MI on echocardiography and an ejection fraction (EF) ≥40% at week 11 were excluded from the study. EF was calculated from end-diastolic and end-systolic volumes obtained with the area-length calculation, on B-mode cine loops recorded in the parasternal long axis view [13]. In week 16, rats were subjected to isoflurane anesthesia, euthanized and organs were removed, weighed and processed for histological quantification and determination of mRNA expression. Left ventricular mass index (LVMI) was calculated as percentage of total body weight (BW, g/100 g). Infarct size was measured on photomicrographs of transverse sections of the heart stained with Sirius Red by dividing the length of the infarct scar by the circumference of the total LV section, traced in the midwall using ImageJ software. Fibrosis was measured on photographs of transverse sections (in remote myocardium of hearts with MI) stained with Sirius Red. Photographs were taken using a polarization filter and analyzed using Adobe Photoshop and ImageJ software. All measurements were performed by an experienced technician blinded to the group allocation. The study protocol was approved by the Ethics Committee on Animal Experiments of the University of Utrecht, Utrecht, The Netherlands.

Immunohistochemistry

Macrophages were stained with an antibody to ED1 and lymphocytes with an antibody to CD3 as previously described [13]. ED1- and CD3-positive cells were counted in the left ventricle. Renal tissue was paraffin embedded and cut into 3-μ sections. Perls' Prussian Blue staining was performed to assess iron deposition. Besides Prussian Blue as a histopathology stain, ferritin immunostaining was used in cardiac and liver tissue to detect the presence of iron. Paraffin sections were deparaffinized with xylene and rehydrated with a series of decreasing ethanol concentrations. Next, an antigen retrieval step using citrate was performed. To block endogenous peroxidise activity, the sections were incubated with 1% BSA. The primary antibody (rabbit anti-ferritin, Sigma-Aldrich) was incubated overnight at 4°C in a 1:50 dilution. A polyclonal biotinylated swine anti-rabbit immunoglobulin antibody

Table 1. Biometric and terminal inflammation variables in the heart at week 15 [13]

	Con (n = 10)	CON + CL (n = 9)	SNX (n = 10)	SNX + CL (n = 7)
BW, g	424±8	426±9	378±11***	361±14###
Hematocrit, ml/ml	0.47±0.01	0.46±0.01	0.42±0.01**	0.43±0.02
Kidney				
Creatinine clearance, ml/min/100 g BW	0.74±0.06	0.67±0.09	0.30±0.03***	0.27±0.04###
Heart				
Cardiac index, ml/min/100 g BW	30±2	17±1***	29±2	14±1$$$
LVMI, g/100 g BW	0.19 (0.19–0.20, n = 8)	0.21 (0.20–0.23, n = 5)	0.35 (0.32–0.39, n = 7)***	0.29 (0.26–0.30, n = 6)$$$, ***
EDV	0.54±0.03, n = 8	0.83±0.11, n = 5***	0.70±0.06, n = 6	0.97±0.05, n = 6$$
CD^{3+} cells in myocardium (per HPF)	1.2 (0.1–2.8)	0.5 (0–1.4)	0.5 (0–1.9)	0.5 (0.2–1.6)
ED^{1+} cells in myocardium (per HPF)	11 (5–15)	16 (12–26)**	11 (7–22)	16 (5–25)

Data as mean ± SEM and as median (range). HPF = High power field. ** $p < 0.01$ vs. CON, *** $p < 0.001$ vs. CON, ### $p < 0.001$ vs. CL, $$ $p < 0.01$ vs. SNX, $$$ $p < 0.001$ vs. SNX. These data were published previously [13].

was used as secondary antibody in a 1:500 dilution for 30 min at RT. Section were subsequently incubated for 30 min with an avidin biotin complex, followed by 3 min of 3.3′-diaminobenzidine. Quantification of ferritin staining was done by means of calculating the reciprocal intensity using ImageJ software, with an average of 10 images per section [14]. In all CL rats, immunohistochemistry was evaluated in regions distant from the infarct.

RNA Isolation and Quantitative Polymerase Chain Reaction

mRNA expression of hepcidin (Hamp; Rn00221783), brain natriuretic peptide (Bnp; Rn00580641), connective tissue growth factor (Ctgf; Rn00573960), Fpn-1 (Slc40a1, Rn00591187), heme oxygenase-1 (Ho-1; Rn00561387) and bone morphogenetic protein 6 (Bmp6; Rn 00432095) in cardiac apical tissue (distant from the infarct in CL rats), and of hepcidin and CCAAT/enhancer binding protein α (C/ebp α; Rn00560963) in liver tissue was assessed by quantitative polymerase chain reaction (qPCR) as described previously [15].

For the real-time PCR using the BIOMARK device (Fluidigm, San Francisco, Calif., USA), RNA was purified from 2 μg total RNA samples using the RNeasy Micro kit (Qiagen, Toronto, Canada) and genomic DNA was removed by RNase-free DNase (Qiagen). Quality and quantity of all RNA samples was using a Bio-Analyzer (Agilent, Santa Clara, Calif., USA). Reverse transcription was carried out on 200–500 ng total RNA per sample using SuperScript® II Reverse Transcriptase and random primers (Thermo Fisher Scientific). The following TaqMan primers were used (all from Thermo Fisher Scientific): calnexin (Canx; Rn01459976_m1), actin-β (Rn00667869_m1), interleukin (IL)-1β (IL-1β; Rn00580432_m1), IL-6 (Rn01410330_m1), caspase 3 (Rn00563902_m1), TNFR-1 (Rn01492348_m1), TLR4 (Rn00569848_m1), C/EBP α (Rn00560963_s1), TNF α (Rn01525860_g1) and TLR2 (Rn01769726_m1). Then, each primer was mixed with DNA suspension buffer (TEKNOVA, Hollister, USA) and each cDNA sample was added. Following the addition of preamp master mix (Fluidigm), 14 circles of pre-amplification were performed on a thermocycler (Bio-Rad, Hercules, Calif., USA). Then, the real-time PCR of each sample in duplicate was performed using the 10 primers in duplicate using a 48*48 IFC and the Fluidigm Biomark HD instrument. The data were collected and analyzed using the Fluidigm Data Collection system, and cycle time (Ct) values were calculated from the software of Biomark Real-Time PCR Analysis. For all samples, at least 4 successful reactions were obtained.

Ct values for all genes were normalized to mean Ct values of Canx (Rn00596877) and β-actin (Rn00667869), which we previously determined to be the 2 most stable housekeeping genes across all groups in both organs using the geNorm program (http://medgen.ugent.be/jvdesomp/genorm/). This produced ΔCt values. Gene expression relative to CON was calculated by subtracting the ΔCt values from the mean ΔCt of the CON group, producing the ΔΔCt values. Statistical analysis was performed on ΔΔCt values, and results were graphed as fold changes compared to CON ($2^{\Delta\Delta Ct}$).

Statistical Analysis

Data were analyzed and graphed using SigmaPlot 12.3 (Systat Software, San Jose, Calif., USA). Data that were not normally distributed were log-transformed to achieve normality. Statistical analysis was performed by 2-way analysis of variance (ANOVA) with the Student-Newman-Keuls post hoc test. Statistical significance was reached with p values <0.05. Univariate linear regression was used to test correlations.

Results

General Results

Longitudinal functional and structural characterization of this model has been published elsewhere [13]. At week 15, rats with SNX had lower BW than rats without SNX, which was not affected by subsequent CL (table 1). SNX caused reduced creatinine clearance ($p < 0.001$ vs. CON)

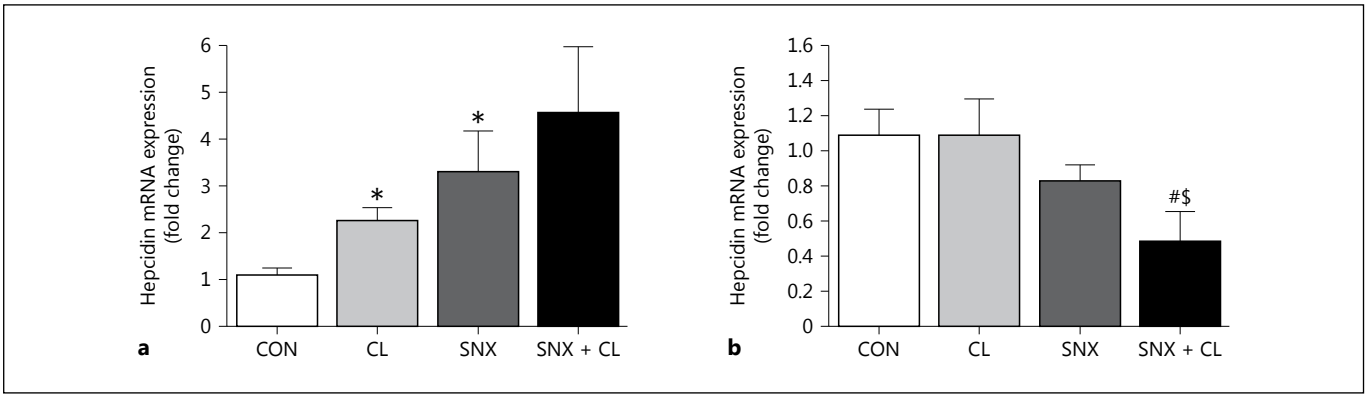

Fig. 1. Hepcidin mRNA expression in heart (**a**) and liver (**b**). ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, * $p < 0.05$ vs. CON, # $p < 0.05$ vs. CL, $ $p < 0.05$ vs. SNX. N heart: CON 9, CL 6, SNX 7, SNX + CL 6. N liver: CON 8, CL 7, SNX 8, SNX + CL 4.

that was not significantly affected by CL in either CL or SNX + CL rats. Given the differences in BW, cardiac parameters are expressed per 100 g BW, where applicable. Cardiac index was lower in SNX + CL than in SNX ($p < 0.01$) and tended to be lower in SNX + CL compared to CL ($p = 0.065$). LVMI increased in SNX rats ($p < 0.001$ vs. CON) but decreased in SNX + CL, while EDV increased only in CL ($p < 0.01$ vs. CON) and SNX + CL rats ($p < 0.01$ vs. SNX).

Immunohistochemistry

The number of inflammatory CD3-positive lymphocytes (T-cells) in the remote myocardium was not different between groups (table 1). Inflammatory ED1-positive cells (monocytes and macrophages) were increased in the remote myocardium after CL compared to CON ($p < 0.01$), irrespective of the presence of SNX.

Hepcidin mRNA Expression in Heart and Liver

To investigate the differential regulation of hepcidin, we assessed hepcidin, that is, *Hamp*, gene expression in both heart and liver tissues. Compared to CON, hepcidin mRNA expression in cardiac tissue was increased 2-fold in CL ($p = 0.03$) and 3-fold in SNX ($p = 0.01$; fig. 1). In contrast, hepatic mRNA expression of hepcidin was unaffected by SNX and CL alone, while it was significantly decreased (50%, $p < 0.05$) in SNX + CL compared to both SNX and CL alone.

Iron Content in Heart and Liver

We used Prussian Blue staining to localize iron storage in heart tissue. In the remote myocardium of CL rats, practically no iron was observed, similar to hearts of non-infarcted (SNX or CON) rats. To extend our observations, we also performed ferritin immunohistochemistry. In line with the Prussian Blue staining, no differences in ferritin content in the remote myocardium of CL and SNX + CL rats as compared to non-infarcted rats were observed (fig. 2a). In liver tissue, there were also no differences seen in ferritin content in CL and SNX + CL rats compared to SNX or CON (fig. 2b).

Cytokine Expression in Cardiac Tissue

To confirm that inflammation was related to cardiac hepcidin expression, we measured cardiac cytokine gene expressions involved in inflammation and apoptosis. Cardiac gene expression of IL-6, IL-1β, caspase 3 and Canx tended to increase in SNX and CL, but the only gene expression reaching statistical significance was IL-1β. Both SNX and CL independently increased cardiac IL-1β gene expression (increase 6-fold, $p < 0.001$ and increase 8-fold, $p < 0.001$, respectively; fig. 3). The combination of both SNX and CL did not further increase cardiac IL-1β gene expression.

Bmp6 mRNA Expression in Cardiac Tissue

The extracellular signaling molecule Bmp6 enhances transcription of the hepatic hepcidin gene in mice [16]. Cardiac Bmp6 mRNA expression level followed the same pattern as cardiac hepcidin mRNA expression. Bmp6 was significantly increased in SNX rats ($p < 0.01$), while the largest increase was seen in SNX + CL rats (fig. 4).

Cardiac BNP and CTGF mRNA Expression

In order to investigate the degree of damage in cardiac tissue, we measured Bnp and Ctgf mRNA expression levels as markers of injury. Cardiac mRNA expression levels of

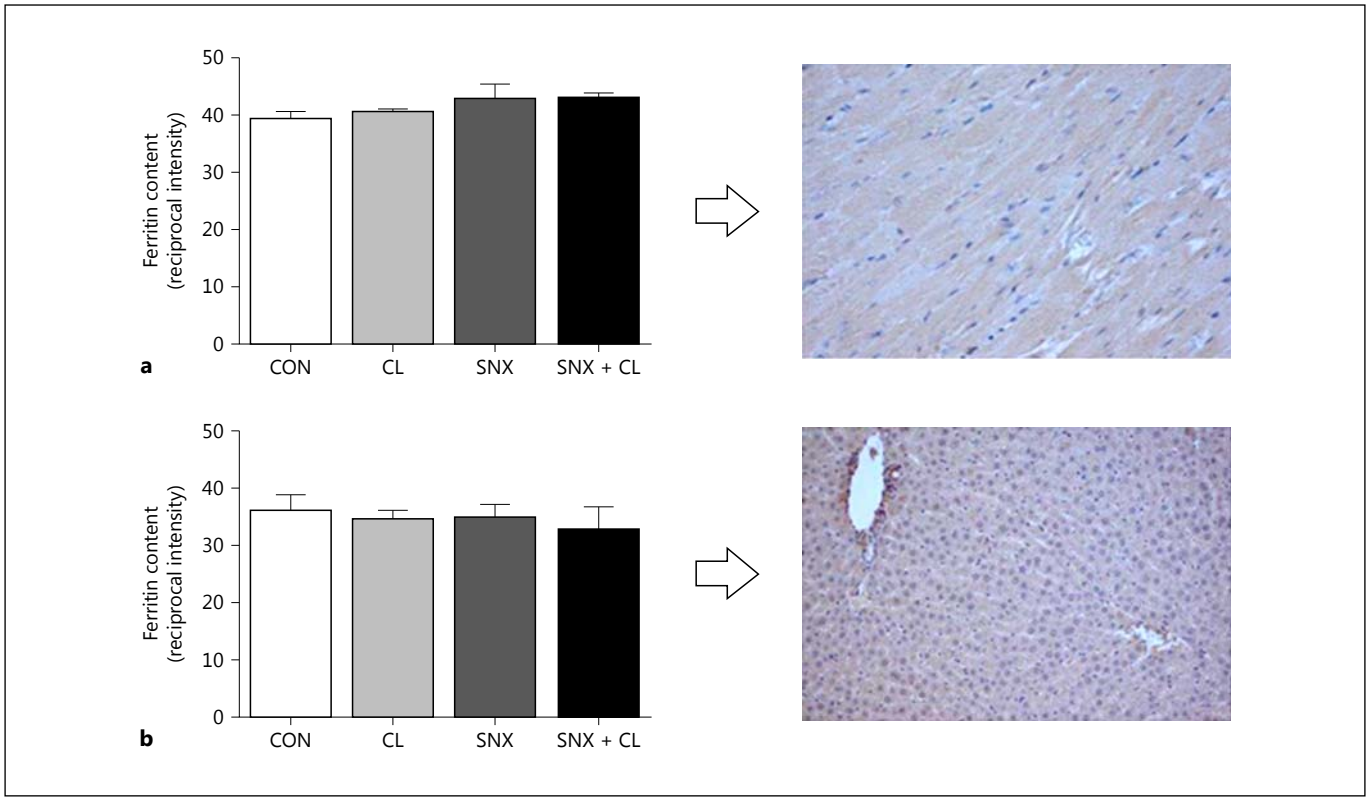

Fig. 2. Quantification of ferritin staining assessed by immunohistochemistry and representative images of ferritin in cardiac tissue (**a**) and liver tissue (**b**). N heart: CON 5, CL 4, SNX 4, SNX + CL 4. N liver: CON 4, CL 4, SNX 5, SNX + CL 3.

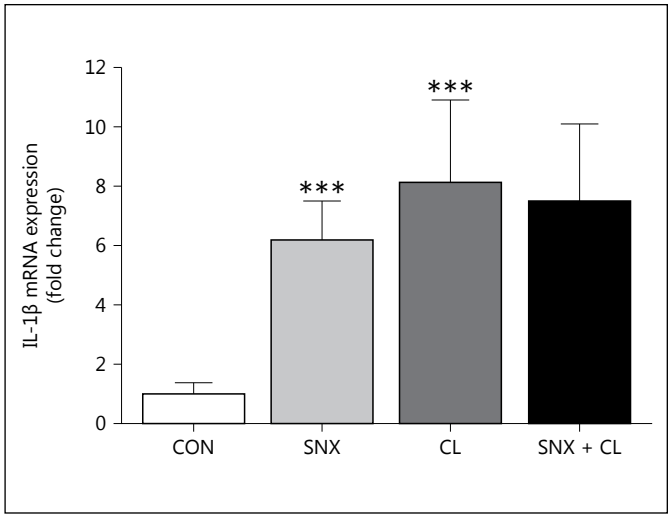

Fig. 3. IL-1β expression in cardiac tissue, assessed by qPCR. ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, *** $p < 0.001$ vs. CON. N: CON 8, CL 5, SNX 12, SNX + CL 6.

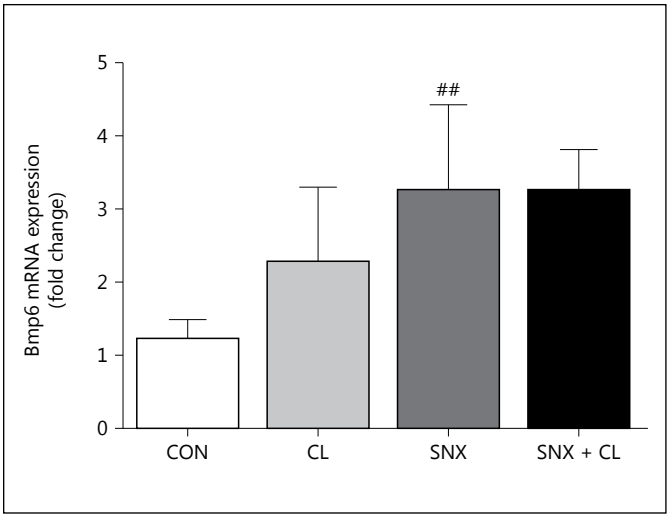

Fig. 4. Bmp6 expression in cardiac tissue, assessed by qPCR. ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, ## $p < 0.01$ vs. CL. N: CON 10, CL 6, SNX 7, SNX + CL 6.

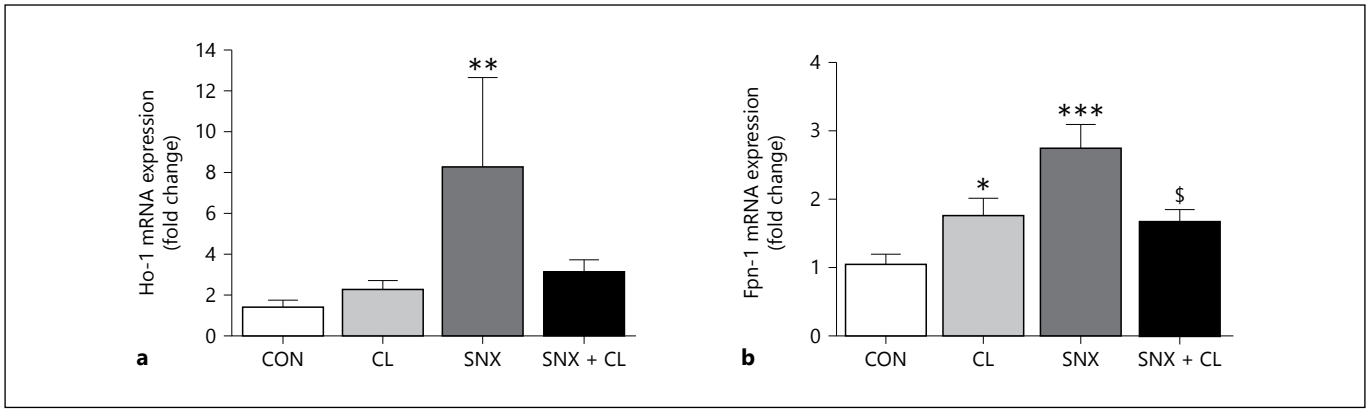

Fig. 5. Ho-1 expression (**a**) and Fpn-1 expression (**b**) in cardiac tissue, assessed by qPCR. ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, * $p < 0.05$ vs. CON, ** $p < 0.01$ vs. CON, *** $p < 0.001$ vs. CON, $^{\$}$ $p < 0.05$ vs. SNX. N: CON 10, CL 6, SNX 7, SNX + CL 6.

Bnp were increased 2.2- and 1.8-fold in SNX and in CL rats, respectively, with a 2.8-fold increase in SNX + CL as reported [13]. Cardiac mRNA expression levels of Ctgf were increased 45- and 18-fold in SNX and in CL rats, respectively, with a 61-fold increase in SNX + CL as reported [13].

Ho-1 and Fpn-1 mRNA Expression in Cardiac Tissue

To get more insight about regulation of iron in the heart, we assessed mRNA expression levels of other proteins involved in iron metabolism such as Ho-1 and Fpn-1 [17]. It is well known that Ho-1 acts as an antioxidant against oxidative injury, which could inhibit the production of reactive oxygen species (ROS) and reduce oxidative damage [18, 19]. Cardiac mRNA expression of Ho-1 was significantly increased in SNX ($p < 0.01$ vs. CON). Ho-1 expression level in SNX + CL tended to be lower than SNX and resembled the level of CL (fig. 5a). Cardiac mRNA expression of Fpt1 showed the same pattern as Ho-1, with a stepwise increase in CL and SNX. Similar to Ho-1, Fpn-1 gene expression in SNX + CL was less than in SNX and similar to CL. Two-way ANOVA showed significant interaction between SNX and CL (fig. 5b; $p = 0.002$).

Hepatic mRNA Expression of C/EBP α

Since C/ebp α plays a key role in regulation of Hamp gene expression in the liver [20], we expected the same pattern for hepatic C/ebp α mRNA expression as for hepatic hepcidin mRNA expression. Indeed, hepatic mRNA expression of C/ebp α decreased progressively across groups with the largest decrease in SNX + CL ($p < 0.05$ vs. CL and $p < 0.05$ vs. SNX; fig. 6).

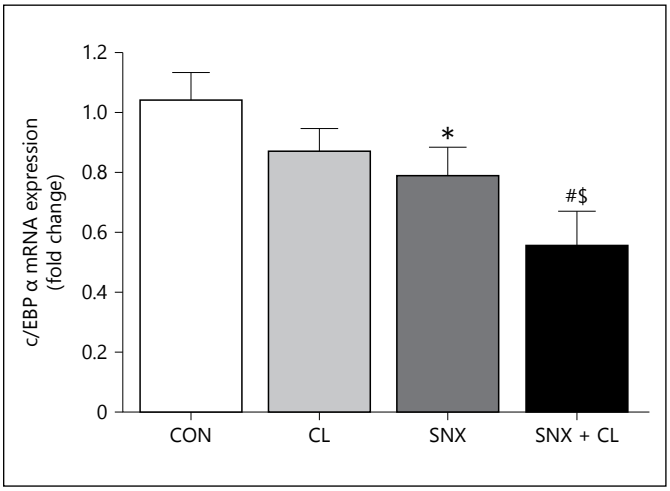

Fig. 6. Hepatic expression of c/EBP α across groups. ΔΔCt values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, * $p < 0.05$ vs. CON, $^{\#}$ $p < 0.05$ vs. CL, $^{\$}$ $p < 0.05$ vs. SNX. N: CON 9, CL 7, SNX 9, SNX + CL 4.

Correlations

In order to investigate possible factors influencing cardiac and hepatic hepcidin expression, correlations were analyzed. Cardiac hepcidin mRNA expression correlated linearly with Bnp mRNA expression ($\beta = 0.73$, $p < 0.001$; fig. 7a) and Ctgf mRNA expression ($\beta = 0.43$, $p = 0.02$; fig. 7b) but not with Bmp6 mRNA expression ($\beta = 0.23$, $p = 0.25$; fig. 7c). Cardiac hepcidin mRNA expression strongly correlated with pro-inflammatory cytokine IL-1β ($\beta = 0.47$, $p = 0.02$; fig. 7d). However, cardiac hepcidin

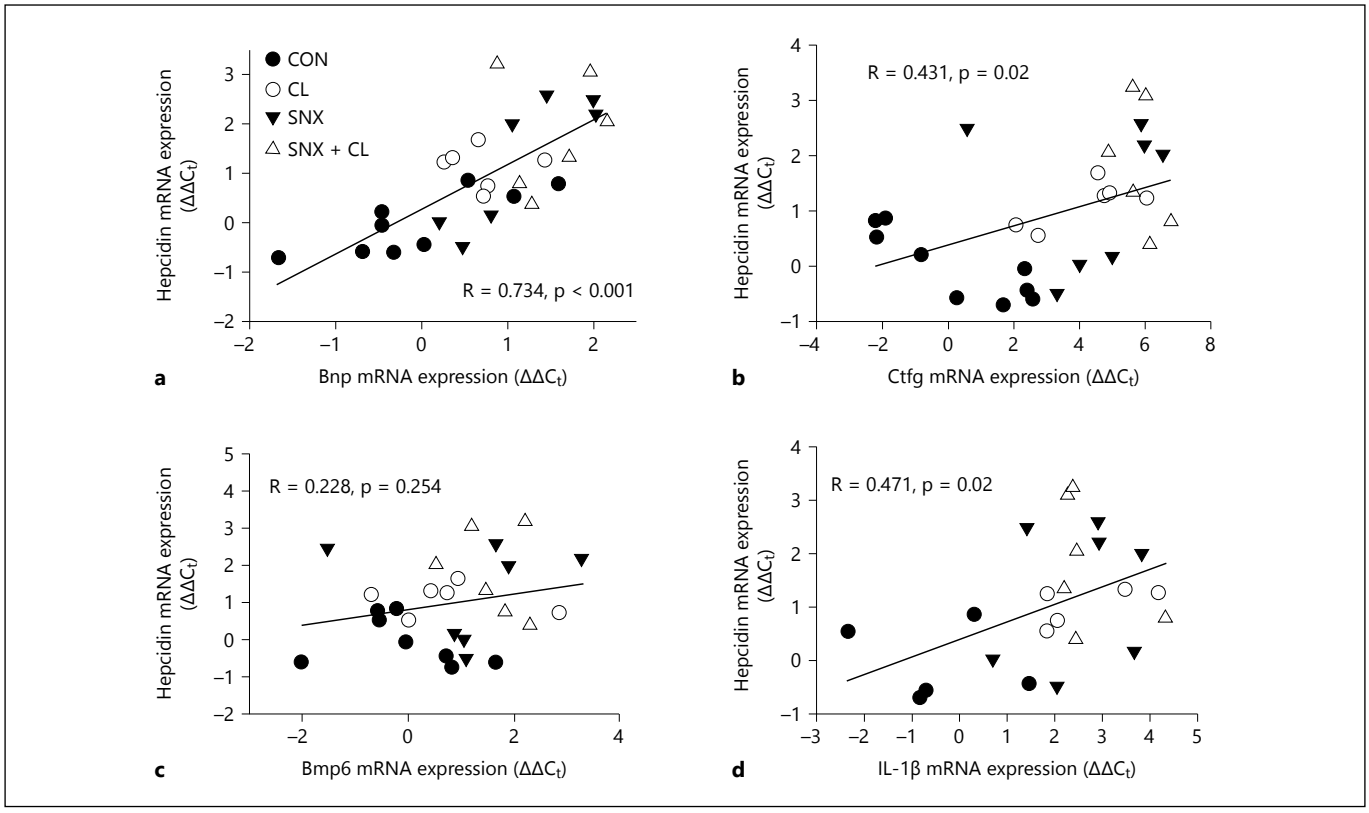

Fig. 7. Correlations between cardiac expression of hepcidin and Bnp (**a**), cardiac hepcidin and Ctgf (**b**), cardiac hepcidin and Bmp6 (**c**) and cardiac hepcidin and IL-1β (**d**). ΔΔCt values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group.

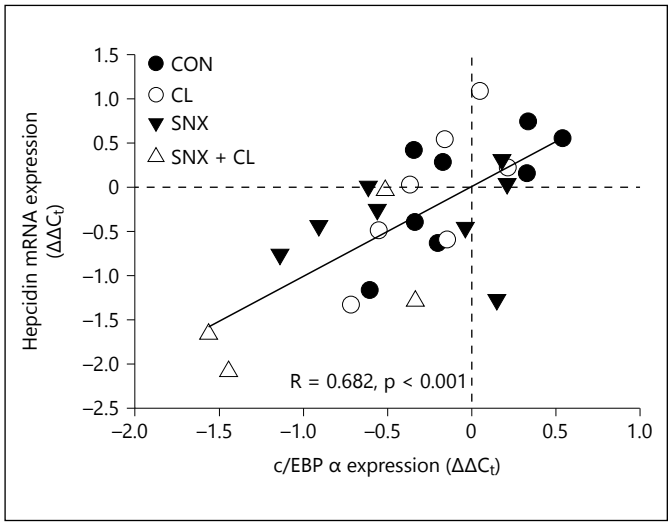

Fig. 8. Correlation between hepatic gene expression of c/EBP α and hepatic hepcidin. ΔΔCt values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group.

mRNA expression did not correlate with cardiac ferritin immunostaining (β = 0.16, p = 0.54).

Hepatic hepcidin mRNA expression correlated with hepatic mRNA expression of the transcription factor C/ebp α (β = 0.68, p < 0.001; fig. 8) but not with ferritin staining (β = 0.14, p = 0.64).

Both LVMI and EDV correlated significantly with cardiac hepcidin mRNA expression (β = 0.53, p < 0.01 and β = 0.62, p < 0.001, respectively; fig. 9a, b). The correlation between cardiac hepcidin mRNA expression and percentage of Sirius Red stained area as a measure of cardiac fibrosis was not significant (β = 0.35, p = 0.1; fig. 9c).

Discussion

The aim of this study was to investigate regulation of hepcidin at tissue level in experimental chronic renocardiac failure. The main finding of this study was that cardiac hepcidin (Hamp) gene expression is significantly in-

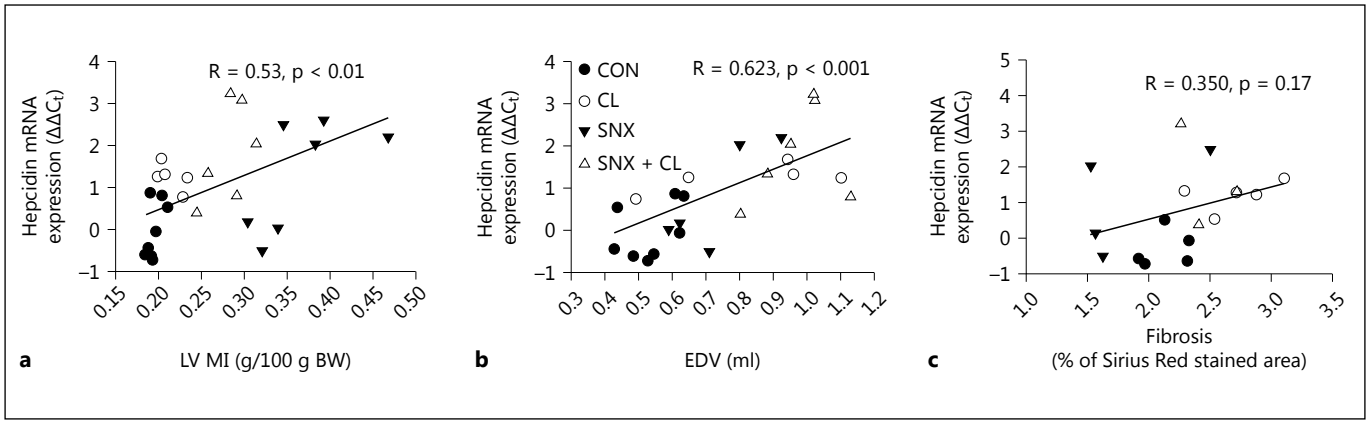

Fig. 9. Correlation between cardiac hepcidin and LVMI (**a**), cardiac hepcidin and EDV (**b**) and cardiac hepcidin and fibrosis (**c**). ΔΔCt values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group.

duced in both local (CL inducing MI) and remote (SNX inducing CKD) injury. Conversely, liver hepcidin gene expression was decreased. Cardiac iron content in non-infarcted tissue remained unchanged in all experimental groups. Thus, cardiac hepcidin expression was increased in response to injury, but no evidence of an association with local iron accumulation was observed.

The increased cardiac expression of hepcidin is in agreement with findings in other models with cardiac injury and myocarditis [8, 9, 21]. In rats, hypoxia and inflammation were shown to upregulate hepcidin expression [8]. In agreement with this finding, hepcidin expression was found to be induced in rat hearts with MI and myocarditis, as well as in human hearts with myocarditis [9]. Furthermore, increased expression of hepcidin was found in the hearts of rats with CKD, which was associated with levels of iron deficiency and anemia [21].

We assessed the mRNA expression of the cardiac damage markers Bnp and Ctgf in myocardial tissue. Bnp is a well-known marker of cardiac dysfunction and has an anti-fibrotic function [22], and Ctgf is a profibrotic cytokine of the Ccn (Cyr61, Ctgf and Nov) family [23] and is suggested to serve as a diagnostic marker of cardiac dysfunction [24, 25]. As shown, there is a progressive increase of Bnp and Ctgf gene expression in the CL, SNX and SNX + CL group. Cardiac hepcidin expression in the SNX + CL group was significantly and positively correlated with cardiac injury based on Bnp and Ctgf but not with ferritin staining. Based on these findings, we postulate that cardiac hepcidin mRNA expression can be induced by local or remote injury and is not necessarily dependent on local iron stores. In the remote myocardium, even though there is no evidence of direct myocardial damage and iron accumulation, hepcidin expression is upregulated.

To confirm our hypothesis that local injury in the heart affects cardiac hepcidin gene expression, we measured the expression of several inflammatory cytokine gene expressions in the heart. Although the cardiac gene expression of IL-6, IL-1β, caspase 3 and Canx tended to increase in SNX and CL, suggesting inflammation and apoptosis, the only gene expression that reached statistical significance is IL-1β. High blood levels of IL-1β have been observed after MI [26] and it is demonstrated that IL-1β induces myocyte hypertrophy [27]. Interestingly, IL-1β also increased in the SNX group. The pathophysiological mechanism of renal failure and increased cardiac IL-1β expression is not elucidated. In experimental models of nephrotic syndrome, glomerular expression of IL-1β is demonstrated [28], probably due to systemic inflammation. Moreira-Rodrigues et al. [29] showed elevated expression of cardiac IL-1β expression in a rat model with nephrotic syndrome, and high levels of IL-1β were measured in the kidneys of rats with spontaneous hypertension [30]. The strong correlation between cardiac hepcidin gene expression and cardiac IL-1β supports the thought that cardiac hepcidin regulation is driven by inflammation instead of iron.

Several authors speculate that hepcidin plays an important protective role against extracellular free radical formation since it decreases the presence of the transmembrane iron exporter ferroportin and thus inhibits cellular iron export [8, 9]. Due to the large amount of iron

in cardiomyocytes, destruction of these cells could result in high iron concentrations in the extracellular space. It is well known that free iron generates damaging ROS and causes various types of injury [31], including myocardial fibrosis. Intracellular iron is stored in ferritin, an important intracellular iron sequestering and storage protein that plays a cytoprotective role against free radical formation by controlling the free cytosolic iron concentration [32]. It could be that during myocardial damage, cardiac hepcidin is upregulated to prevent iron release from the intact cells. However, in contrast to this possible mechanism, in our study, cardiac ferritin content was not related to hepcidin expression. Our data suggest that hepcidin gene expression is influenced by local injury not related to local iron turn over; there is enhanced cardiac hepcidin mRNA expression in the absence of changed cardiac ferritin content. Besides, liver expression of Bmp6 is transcriptionally regulated by iron – overload of iron increases Bmp6 expression, iron deficiency represses Bmp6 expression [33]. However, in our study, cardiac BMP6 mRNA expression was significantly increased in SNX and SNX + CL rats, but no differences in iron content in the remote myocardium was observed. This could implicate that in the heart, it is not iron, but other stimuli such as injury, which stimulate Bmp6 expression. Bmp6 expression could be part of a compensatory mechanism in the heart to stimulate cardiomyocyte hypertrophy [34]. Korf-Klingebiel et al. [34] found that recombinant Bmp6 (even in low concentrations) resulted in a robust increase in cell size and protein synthesis in neonatal and adult cardiomyocytes, respectively.

In contrast to hepcidin and the damage markers Bnp and Ctgf, and contrary to our expectations, Fpn-1 and Ho-1 mRNA expression showed a different pattern. For Fpn-1 and Ho-1, there was an interaction between SNX and CL, in that the presence of both eliminated the SNX effect. The basis for this observation is not clear. Although it is widely assumed that hepcidin induces ferroportin internalization and degradation, this relation is not as clear as it seems. In monocytes of HD patients, no correlation between serum hepcidin and ferroportin was found [35]. This suggests that factors other than hepcidin also affect ferroportin levels and internalization and that degradation of ferroportin can take place in a hepcidin-independent manner. The fact that this relation is absent in cell types in which hepcidin is thought to play its classical role (ferroportin internalization and degradation) supports our result regarding the lack of such a relation at the mRNA level in the heart in our cardiorenal syndrome model.

Unexpectedly, combined SNX and CL showed a decrease in hepatic hepcidin mRNA expression. Both cardiac and renal failure is associated with an increase of inflammatory cytokines, which could have resulted in an upregulation of hepatic hepcidin gene expression [36]. However, several studies reported low serum hepcidin levels in patients with chronic heart failure and anemia [37, 38]. One possible explanation is that iron deficiency, even in the presence of increased cytokines, leads to diminished levels of hepcidin in conditions of heart failure and anemia. Although in our rodent model only mild anemia was present, anemia and increased erythropoiesis (i.e., production of red blood cells) could downregulate the synthesis of hepcidin mRNA [39]. Unfortunately, we did not measure circulating inflammatory cytokines and iron markers to confirm this hypothesis.

In agreement with this finding, we showed a correlation between mRNA expressions of C/ebp α and hepcidin in the liver, in the sense that both were reduced. C/ebp α is a transcription factor in the liver that has a positive effect on Hamp promoter activity [20]. Alcoholic and viral liver cell damage both reduce hepcidin expression, a reduction which is mediated by ROS via C/ebp α [40, 41]. Reduction in hepatic C/ebp α mRNA expression in renocardiac failure may associate with hepatic oxidative stress [42].

LVMI increased in SNX rats and decreased in SNX + CL rats, most probably due to the loss of cardiomyocytes after cardiac injury. As a measure of cardiac preload and increased filling pressures, EDV increased in CL and SNX + CL rats. These results are in line with previous findings that LVMI increases during renal and cardiac failure, the predominant pattern being eccentric left ventricular hypertrophy [43, 44]. In accordance with previous research [45], the significant correlation between cardiac hepcidin mRNA expression and LVMI and EDV suggests that hepcidin plays a role in the hypertrophic response in cardiomyocytes. Once again, this emphasizes the role of hepcidin in cardiac damage.

Given the fact that liver hepcidin is only regulated at the transcriptional level, we used HAMP mRNA levels to detect cardiac hepcidin. Accordingly, we studied the stimulatory effect of cardiac injury on cardiac hepcidin expression rather than the reverse.

In conclusion, cardiac expression of hepcidin is differentially regulated in this rat model of renocardiac failure from its primary source of production, the liver. Moreover, our data suggest a role for injury rather than iron as a driving force for cardiac hepcidin expression in experimental renocardiac failure. Future animal and cell exper-

iments should be developed to study the mechanisms of systemic and local hepcidin production and action in different tissues. Elucidating the pathophysiological mechanisms of local hepcidin regulation in patients with CKD and/or heart failure may help designing new therapeutic approaches.

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Disclosure Statement

The authors have no conflict of interest to declare.

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